

## Research Article

# Hemotoxicity Induced by Chronic Chlorpyrifos Exposure in Wistar Rats: Mitigating Effect of Vitamin C

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Received 10 November 2010; Accepted 23 February 2011

Academic Editor: José Cerón

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The study evaluated the ameliorative effect of vitamin C on chronic chlorpyrifos-induced hematological alterations in Wistar rats. Twenty adult male rats divided into 4 groups of 5 animals each were exposed to the following regimens: group I (S/oil) was administered soya oil (2 mL/kg b.w.), while group II (VC) was given vitamin C (100 mg/kg b.w.); group III was dosed with CPF (10.6 mg/kg b.w.); group IV was pretreated with vitamin C (100 mg/kg) and then exposed to CPF (10.6 mg/kg b.w.), 30 minutes later. The regimens were administered by oral gavage once daily for a period of 17 weeks. Blood samples collected at the end of the study revealed reduction in the levels of pack cell volume, hemoglobin, red blood cells, leukocytes (attributed to neutropenia, lymphopenia, and monocytopenia), and platelets in the CPF group, which were ameliorated in the vitamin C- pretreated group. The elevated values of malonaldehyde, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, and neutrophil/lymphocyte ratio in the CPF group were restored in those pretreated with vitamin C. The study has shown that chronic CPF-induced adversity on hematological parameters of Wistar rats was mitigated by pretreatment with vitamin C.

## 1. Introduction

Organophosphate (OP) insecticides are used in the agricultural and domestic pest control [1], accounting for 50% of the global insecticidal use [2]. Their use is, however, accompanied by widespread toxicity in nontarget organisms, including man. Chlorpyrifos (CPF) is one of the most widely used OP insecticides until 2000 when the United States Environmental Protection Agency restricted some of its domestic uses due to its toxicity. Despite this, CPF remains one of the most widely used OP insecticides. Anemia and alteration in other hematological parameters have been recorded following repeated CPF exposure [3, 4]. Although the mechanism of acute CPF toxicity involves acetylcholinesterase (AChE) inhibition, other mechanisms unrelated to AChE inhibition, including the induction of oxidative stress, have been implicated [4–8]. As a lipophilic molecule, CPF easily passes through the cells into the cytoplasm [9]. Once inside the cell, CPF induces damage to the cellular molecules [10].

Oxidative damage primarily occurs through production of reactive oxygen species (ROS) which causes damage to macromolecules such as lipids, proteins, and DNA. Under normal circumstances, the body copes with oxidative assault through the repair of the damage or the invocation of the indigenous antioxidant enzymatic and nonenzymatic systems to reduce the pro-oxidation states. However, in situation of increased and accelerated oxidative challenge by CPF as previously reported [4–8], the natural antioxidant mechanisms are overwhelmed thereby resulting in damage. Therefore, supplementation with exogenous source of antioxidant is likely to reduce the oxidative burden, hence tissue damage. Vitamin C is one of the most widely available and affordable nonenzymatic antioxidant molecules that have been used to mitigate oxidative damage. It is an important water-soluble antioxidant in biological fluids [11, 12]. It readily scavenges physiological ROS such as superoxide, hydroxyl, and aqueous peroxy radicals, as well as nonradical species such as singlet oxygen and ozone, as well as reactive

nitrogen species (RNS) such as peroxyxynitrite, nitrosating species ( $N_2O_3/N_2O_4$ ), nitroxide radicals, and nitrogen dioxide [13, 14]. The reduction in CPF-induced toxicity following vitamin C supplementation has been reported previously [6, 7]. The aim of the present study is therefore to evaluate the mitigating effect of vitamin C on hematological changes induced by chronic CPF exposure in Wistar rats.

## 2. Materials and Methods

**2.1. Animals and Housing.** Twenty young adult male Wistar rats weighing 95–110 g were obtained from the Laboratory Animal Unit of the Department of Veterinary Physiology and Pharmacology, Ahmadu Bello University, Zaria, Nigeria. They were housed in metal cages and fed on standard rat chow, and water was provided *ad libitum*. The animals were allowed to acclimatize for at least one week. The housing and management of the animals and the experimental protocols were conducted as stipulated in the Guide for Care and Use of Laboratory Animals [15].

**2.2. Chemicals.** Commercial grade CPF (Termicot, 20% EC, Sabero Organics, Gujarat, India) was dissolved in soya oil (Grand Cereal, Jos, Nigeria), while each tablet of vitamin C, Med Vit C (100 mg/tablet; Dol-Med Laboratories Limited, Lagos, Nigeria), was dissolved in 1 mL of distilled water to obtain 100 mg/mL suspension, just prior to its daily administration.

**2.3. Experimental Protocol.** The rats were weighed using digital weighing balance and then assigned randomly into 4 groups of 5 rats in each group. Rats in group I served as the control group (S/oil) and were given only soya oil (2 mL/kg b.w.), while those in group II (VC) were dosed with vitamin C (100 mg/kg b.w.). Rats in group III (CPF) were administered with CPF only (10.6 mg/kg b.w.  $\sim$ 1/8th LD<sub>50</sub> of 85 mg/kg) [16], while those in group IV (VC+CPF) were pretreated with vitamin C (100 mg/kg) and then dosed with CPF (10.6 mg/kg b.w.), 30 min later. The different regimens were administered once daily by oral gavage for a period of 17 weeks. At the end of the study period, the rats were sacrificed by severing the jugular vein after light ether anesthesia.

**2.4. Hematological Evaluation.** Two milliliters of blood collected into heparinized sample bottles were analyzed for hematological parameters such as pack cell volume (PCV), hemoglobin (Hb), total red blood cells (RBCs), mean cell volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), total white blood cell (WBC), and total platelets count using an automatic hematological assay analyzer, Advia 60 Hematology system (Bayer Diagnostics Europe Ltd, Ireland). Blood smears were also stained with Giemsa for absolute differential WBC count [17], while the neutrophil-lymphocyte ratio was calculated.

**2.5. Evaluation of Erythrocytes Malonaldehyde Concentration.** The erythrocyte malonaldehyde (MDA) concentration, as

a marker of lipid peroxidation, was determined by the double heating method of Draper and Hadley [18], as we described previously [4, 6]. The principle of the method was spectrophotometric measurement of the colour produced during the reaction of thiobarbituric acid (TBA) with MDA. One milliliter of heparinized blood samples obtained from each animal was centrifuged at 600 g and the plasma discarded. Erythrocyte packets were prepared by washing erythrocytes three times in cold isotonic saline (0.9% w/v). The washed erythrocytes were used to analyze for MDA concentrations. Briefly, 2.5 mL of 100 g/L trichloroacetic acid was added to 0.5 mL of erythrocytes in a centrifuge tube and placed in a boiling water bath for 15 min. After cooling in tap water, the mixture was centrifuged at  $1000 \times g$  for 10 min, and 2 mL of the supernatant was added to 1 mL of 6.7 g/L TBA solution in a test tube and placed in a boiling water bath for 15 min. The solution was then cooled in tap water, and its absorbance measured using a UV spectrophotometer (Jenway, 6405 model, Japan) at 532 nm. The concentration of MDA was calculated by the absorbance coefficient of MDA-TBA complex,  $1.56 \times 10^5 \text{ cm}^{-1} \text{ M}^{-1}$ , and expressed in nanomoles per gram of hemoglobin. The hemoglobin concentration was determined using the method of Dacie and Lewis [19].

**2.6. Statistical Analysis.** Values obtained as mean  $\pm$  SEM were subjected to one-way analysis of variance (ANOVA) followed by Tukey test using GraphPad Prism version 4.0 for windows from GraphPad Software, San Diego, California, USA). Values of  $P < .05$  were considered significant.

## 3. Results

**3.1. Effects of Treatments on Pack Cell Volume.** The PCV recorded for rats in the CPF group was significantly lower compared to either the S/oil ( $P < .05$ ) or the VC ( $P < .01$ ) group. There was no significant change in the PCV of rats in the VC+CPF group compared to any of the other groups (Table 1).

**3.2. Effect of Treatments on Hemoglobin Concentration.** The Hb concentration was significantly lower in the CPF group compared to either the S/oil ( $P < .05$ ) or the VC ( $P < .01$ ) group. There was no significant difference ( $P > .05$ ) in the Hb of VC+CPF group compared to either the S/oil, VC, or CPF group (Table 1).

**3.3. Effect of Treatments on Total Red Blood Cell Concentration.** A significantly lower RBC concentration was recorded in the CPF group compared to either the S/oil ( $P < .01$ ), VC ( $P < .01$ ), or VC+CPF ( $P < .05$ ) group. The RBC concentration in VC+CPF group was significantly lower ( $P < .05$ ) compared to those recorded in the VC group, but was marginally higher than in the CPF group (Table 1).

**3.4. Effect of Treatments on Red Blood Cell Indices.** The effect of treatments on MCV, MCH, and MCHC is shown in Table 1. The MCV and MCH in the CPF group were

TABLE 1: Effect of chronic exposure to soya oil (S/oil), vitamin C (VC), and/or chlorpyrifos (CPF) on pack cell volume (PCV), red blood cell (RBC) and hemoglobin (Hb) concentrations, and erythrocyte indices in Wistar rats.

Parameters	S/oil	VC	CPF	VC+CPF
PCV (%)	42 ± 1.6	44 ± 1.3	35 ± 1.2 <sup>ab</sup>	40 ± 0.95
Hb (g/dL)	14 ± 0.53	15 ± 0.64	12 ± 0.37 <sup>ab</sup>	13 ± 0.31
RBC count (×10 <sup>12</sup> /L)	5.8 ± 0.49	6.8 ± 0.3	3.7 ± 0.15 <sup>abc</sup>	5.1 ± 0.07 <sup>d</sup>
MCV (fL/cell)	79 ± 11	63 ± 6.0	9.3 ± 3.1 <sup>b</sup>	7.9 ± 3.0
MCH (pg/cell)	25 ± 2.7	24 ± 2.2	33 ± 2.1 <sup>b</sup>	26 ± 0.84
MCHC (g/dL)	33 ± 1.5	33 ± 2.2	34 ± 2.0	33 ± 0.8

<sup>a</sup>*P* < .01 versus soya oil group; <sup>b</sup>*P* < .01 versus vitamin C group; <sup>c</sup>*P* < .05 versus vitamin C+chlorpyrifos group; <sup>d</sup>*P* < .01 versus vitamin C group. Values are mean ± SEM of 5 animals per group. NB-MCV: mean cell volume; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration.

significantly elevated (*P* < .05) compared to those recorded in the VC group. There was no significant change (*P* > .05) in MCHC in between the groups. Anisocytosis was also observed in the CPF group compared to normocytosis in the other groups.

**3.5. Effect of Treatments on Total and Absolute Differential White Blood Cell Counts.** There was a significant decrease (*P* < .01) in the WBC counts of CPF group compared to either S/oil or VC group. The WBC concentration in the VC+CPF group was significantly lower (*P* < .05) compared to those recorded in the VC group (Table 2).

The neutrophil count in the CPF group was significantly lower compared to the S/oil (*P* < .01), VC (*P* < .01) and VC+CPF (*P* < .05) groups, respectively. The neutrophil count in the VC+CPF group was significantly lower (*P* < .01) compared to either the S/oil or VC group. The lymphocyte count in the CPF group was significantly lower (*P* < .01) compared to either the S/oil, VC, or VC+CPF group. The lymphocyte count of VC+CPF group was significantly lower compared to either S/oil (*P* < .05) or VC (*P* < .01) group. The monocyte count in the CPF group was significantly lower in the CPF group compared to either S/oil (*P* < .05) or VC (*P* < .01) group. The monocyte count in the VC+CPF group was significantly lower (*P* < .05) compared to the VC group (Table 2).

**3.6. Effect of Treatments on Neutrophil/Lymphocyte Ratio.** The neutrophil/lymphocyte ratio in the CPF group was significantly higher compared to either the S/oil (*P* < .05), VC (*P* < .05), or VC+CPF (*P* < .01) group. The neutrophil/lymphocyte ratio of VC+CPF group was not significantly different (*P* > .05) from those obtained in either the S/oil or VC group (Table 2).

**3.7. Effect of Treatments on Platelet Count.** The platelet count in the CPF group was significantly lower compared to either the S/oil (*P* < .01), VC (*P* < .01), or VC+CPF (*P* < .05)

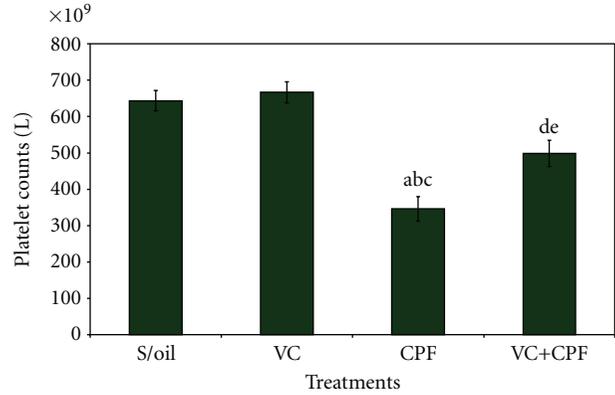


FIGURE 1: Effect of chronic exposure to soya oil (S/oil), vitamin C (VC), and/or chlorpyrifos (CPF) on platelet counts in Wistar rats. <sup>a</sup>*P* < .01 versus soya oil group; <sup>b</sup>*P* < .01 versus vitamin C group; <sup>c</sup>*P* < .05 versus vitamin C+chlorpyrifos group; <sup>d</sup>*P* < .05 versus soya oil group; <sup>e</sup>*P* < .05 versus vitamin C group. Values are mean ± SEM of 5 animals per group.

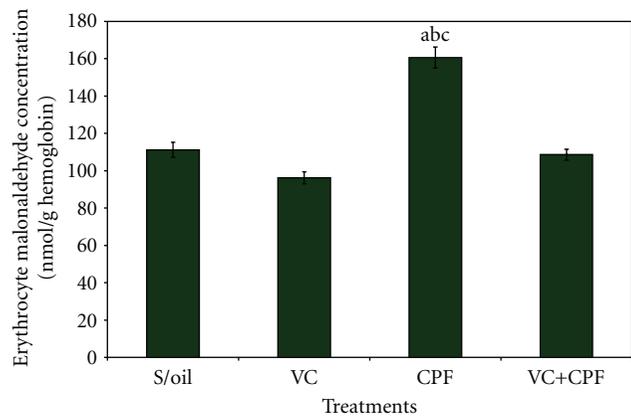


FIGURE 2: Effect of chronic exposure to soya oil (S/oil), vitamin C (VC), and/or chlorpyrifos (CPF) on erythrocytes malonaldehyde concentration. <sup>abc</sup>*P* < .01 versus soya oil, vitamin C, and vitamin C+chlorpyrifos groups, respectively.

group. The platelet count recorded in the VC+CPF group was significantly lower (*P* < .05) relative to either the S/oil or VC group (Figure 1).

**3.8. Effect of Treatments on Erythrocyte Malonaldehyde Concentration.** The erythrocyte MDA concentration in the CPF group was significantly higher (*P* < .01) compared to those obtained in the soya oil, VC, and VC+CPF groups, respectively. The MDA concentration in VC+CPF group was not significantly different from those recorded in either the VC or the S/oil group (Figure 2).

#### 4. Discussion

The low hematological parameters of PCV, Hb, and RBC show that chronic CPF administration causes anemia. This

TABLE 2: Effect of chronic exposure to soya oil (S/oil), vitamin C (VC), and/or chlorpyrifos (CPF) on absolute total and differential leukocyte count in Wistar rats.

Parameters ( $\times 10^9/L$ )	S/oil	VC	CPF	VC+CPF
Total leukocyte count ( $\times 10^9/L$ )	$9.9 \pm 0.81^a$	$9.9 \pm 0.89$	$5.4 \pm 0.37^{ab}$	$8.4 \pm 0.5^i$
Neutrophils count ( $\times 10^9/L$ )	$4.4 \pm 0.12$	$4.9 \pm 0.19$	$2.7 \pm 0.15^{abc}$	$3.4 \pm 0.16^{de}$
Lymphocytes count ( $\times 10^9/L$ )	$5.3 \pm 0.18$	$5.9 \pm 0.16$	$3.0 \pm 0.13^{abf}$	$4.5 \pm 0.19^{eg}$
Monocytes count ( $\times 10^9/L$ )	$0.07 \pm 0.01$	$0.09 \pm 0.02$	$0.0 \pm 0.0^{ch}$	$0.03 \pm 0.01^i$
Band cells count ( $\times 10^9/L$ )	$0.0 \pm 0.0$	$0.0 \pm 0.0$	$0.0 \pm 0.0$	$0.01 \pm 0.003$
Neutrophil:Lymphocyte ratio	$0.84 \pm 0.05$	$0.82 \pm 0.09$	$1.0 \pm 0.0^{abc}$	$0.76 \pm 0.04$

<sup>a</sup> $P < .01$  versus soya oil group; <sup>b</sup> $P < .01$  versus vitamin C group; <sup>c</sup> $P < .05$  versus vitamin C+chlorpyrifos group; <sup>d</sup> $P < .01$  versus Soya oil group; <sup>e</sup> $P < .01$  versus Vitamin C group; <sup>f</sup> $P < .01$  versus vitamin C+chlorpyrifos group; <sup>g</sup> $P < .05$  versus Soya oil group; <sup>h</sup> $P < .05$  versus soya oil group; <sup>i</sup> $P < .05$  versus vitamin C group. Values are mean  $\pm$  SEM of 5 animals per group.

agreed with earlier findings [3, 4, 20, 21]. Goel et al. [3] attributed the anemia to the ability of CPF to reduce serum iron concentration, thereby compromising the synthesis of Hb. The anemia may also be related to interference with Hb synthesis and shortening of RBC lifespan [22]. We have earlier shown that chronic CPF exposure causes increased erythrocyte fragility, partly due to increased lipoperoxidation of the erythrocyte membranes [4, 7, 8]. The increased lipoperoxidation in the CPF group, reflected by significant MDA concentration, may have caused increased vulnerability of the RBC to destruction, but may directly destroy the erythrocytes thereby leading to anemia. MDA is a major oxidation product of peroxidized polyunsaturated fatty acids (PUFAs), and increased MDA content is an important indicator of lipid peroxidation [23].

The RBC is susceptible to lipoperoxidative changes because of its direct association with molecular oxygen, high content of metal ions catalyzing oxidative reactions, and availability of high amount of PUFAs, which are susceptible to lipid peroxidation. Inability to repair membrane damage and regenerate due to lack of nucleus and poor antioxidant enzymes composition of the plasma medium in which they are bathed [24, 25] are some of the other factors responsible for the increased vulnerability of RBC to lipoperoxidation. Therefore, CPF-induced oxidative damage to the erythrocyte membrane may have contributed to the anemia recorded in the CPF group. This is because the process of lipid peroxidation impairs the functions and homeostasis of the erythrocyte membranes through decrease in hydrophobic characteristics of bilayer membrane, and altering the affinity and interaction of proteins and lipids [26]. ROS can equally affect the proteins resulting in modification of enzymes activity, and damage to the membrane transport proteins may produce disturbed cellular ionic homeostasis, leading to alterations in intracellular calcium and potassium that triggers a series of changes in the cell [27]. ROS can directly affect the conformation and/or activities of all sulfhydryl-containing molecules, by oxidation of their thiol moiety [28, 29]. The combined effect of these ROS-triggered cellular changes may eventually lead to cellular dysfunction and ultimate destruction.

Anisocytosis observed in the CPF group in the present study had also been recorded in earlier studies [3, 4].

The increased MCV may reflect the presence of immature RBCs in the peripheral blood, perhaps arising from the body compensatory mechanism to cater for the CPF-induced deficit in RBC concentration. The increased presence of immature RBCs may be similarly responsible for the anisocytosis observed in the CPF group. The significant increase in MCH in the CPF group shows that the amount of Hb in this group is high, while the apparently normal MCHC indicates normal Hb concentration. Therefore, the OP insecticide can be said to induce macrocytic anemia.

The lack of significant increase in PCV and concentrations of RBCs and Hb recorded in group pretreated with vitamin C when compared to the S/oil or VC group was an indication of the attenuation of CPF-evoked anemia by the antioxidant vitamin. In its reduced form, vitamin C has been shown to improve the absorption of iron from the gut [30, 31], thereby increasing its serum concentration of iron essential for heme synthesis. This is by facilitating the reduction of ferric iron to the ferrous form [32]. Besides, vitamin C has also been shown to be beneficial in the management of anemia [33]. Furthermore, the amelioration of the anemia in the group pretreated with vitamin C may be due to reduction in lipoperoxidative damage to the erythrocyte membrane as demonstrated by its low MDA concentration in the present study. Similarly, the low erythrocyte fragility observed in our earlier study following vitamin C supplementation of rats chronically exposed to CPF [7] may have contributed to the mitigation of anemia in the present study.

The present study also revealed leucopenia apparently due to lymphopenia, neutropenia, and monocytopenia in the CPF group. Previous studies have attributed CPF-induced leucopenia to neutropenia [6] and lymphopenia [3, 4]. Ambali et al. [4] reported neutrophilia following CPF exposure, in contrary to neutropenia recorded in the present study. Levine et al. [34] attributed monocytopenia recorded in workers exposed to OP to inhibition of a monocyte esterase, [ $\alpha$ ]-naphthyl butyrate esterase. Many pesticides have been shown to induce immunotoxicity either via the induction of apoptosis or necrosis [35, 36]. CPF exposure has been shown to induce immunotoxicity via the induction of apoptosis partly mediated through the activation of caspase 3 [37]. Chronic CPF exposure has been associated with

abnormality of the immune system including depression of T-lymphocytes [38]. Immunotoxicity in OPs has been associated with either inhibition of serine hydrolases or esterases in components of the immune system, through oxidative damage to immune organs, or by modulation of signal transduction pathways controlling immune functions [39]. Free radical-induced oxidative damage that has been widely implicated in the molecular mechanism of CPF cytotoxicity is an initiator of apoptosis [35, 40], which may have been involved in the depletion of the components of the WBC in the group exposed to the OP in the present study.

Vitamin C pretreatment was able to mitigate the CPF-induced immunotoxicity by restoring the concentration of leukocytes and its components. The ability of vitamin C to restore subchronic CPF-induced leucopenia has been demonstrated in our earlier study [6]. Vitamin C has been shown to enhance immune response via numerous mechanisms, including lymphocytes proliferation [41, 42]. Besides, the antioxidant function of the vitamin has been shown to inhibit apoptosis [43, 44].

The increase in the neutrophil/lymphocyte ratio (NLR) in the CPF group recorded in the present study has been reported previously in our laboratory following subchronic CPF exposure [7]. NLR provides an indication of inflammatory status in patients [45] and has been used as a prognostic factor in predicting clinical outcomes of a disease process and in the situation of increased stress or inflammation [45–47]. NLR correlates well with the magnitude of total leukocyte response and may provide a parameter that is more sensitive than the total leukocyte count in a disease process [48]. The elevated NLR in the CPF group in this study is a demonstration of ongoing stress and inflammatory process in rats from this group, predicating bad clinical outcomes. The NLR in the group pretreated with vitamin C was not significantly different from those observed in the group administered either soya oil or vitamin C only, indicating amelioration of CPF-induced stress and inflammatory process in the group, partly due to protection from oxidative damage by the antioxidant vitamin.

The significant decrease in platelet count in the CPF group shows that chronic exposure to the insecticide caused thrombocytopenia. This finding contradicted what we reported earlier [4] that recorded thrombocytosis following subchronic CPF exposure. The reason for the discrepancy is not clear but may be related to the duration of exposure. Thrombocytopenia may be related to CPF-induced oxidative damage to the platelet membranes. A direct relationship between oxidative stress and thrombocytopenia has been demonstrated in patients infected with malaria parasites [49]. The significant improvement in the level of thrombocytes in group pretreated with the vitamin further underscored the role of oxidative stress in CPF-induced thrombocytopenia.

In conclusion, the present study has shown that vitamin C pretreatment ameliorated the chronic CPF-induced hemotoxicity in Wistar rats. This may be partly due to free radical scavenging properties of the antioxidant vitamin, which attenuated CPF-evoked lipoperoxidation to the blood cellular constituents. However, the other nonantioxidant role

of vitamin C may have also complemented this antioxidant mechanism of cytoprotection. Therefore, the results of this study give an indication that vitamin C supplementation may mitigate hemotoxicity in individuals who are at risk of prolonged CPF exposure.

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